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RESEARCH PAPER

Isolation and characterization of *Viviparous-1* genes in wheat cultivars with distinct ABA sensitivity and pre-harvest sprouting tolerance

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Abstract

Pre-harvest sprouting (PHS) of wheat reduces the quality and economic value of grain, and increasing PHS tolerance is one of the most important traits in wheat breeding. Two new *Vp-1B* alleles related to PHS tolerance were identified on the 3BL chromosome of bread wheat and were designated *Vp-1Bb* and *Vp-1Bc*. Sequence analysis showed that *Vp-1Bb* has a 193 bp insertion and *Vp-1Bc* has a 83 bp deletion located in the third intron region of the *Vp-1B* gene, and that they shared 95.43% and 97.89% similarity, respectively, with the sequence of AJ400713 (*Vp-1Ba*) at the nucleotide level. Their sequences were deposited in the GenBank under the accession numbers DQ517493 and DQ517494. Semi-quantitative RT-PCR analysis showed that alternatively spliced transcripts of the *Vp-1A*, *Vp-1B*, and *Vp-1D* homologues were present and there were no differences in the splicing patterns or abundances of *Vp-1A* and *Vp-1D* from embryos 35 d after pollination between PHS-tolerant and -susceptible cultivars. Although *Vp-1Ba, Vp-1Bb*, and *Vp-1Bc* could each produce a set of transcripts, only one was correctly spliced and had the capacity to encode the full-length VP1 protein and was more highly expressed with *Vp-1Bb* and *Vp-1Bc* than with *Vp-1Ba*. Comparison of the expression patterns of *Vp-1Ba, Vp-1Bb*, and *Vp-1Bc* on different days after pollination also revealed that the expression of these genes was developmentally regulated. Furthermore, genotypes with different levels of tolerance to PHS respond differently to ABA exposure and differences in transcript levels of *Vp-1Ba*, *Vp-1Bb*, and *Vp-1Bc* were observed after ABA treatment. The results indicated that insertion or deletion in the third intron region might affect the expression of the *Vp-1B* gene and its sensitivity to ABA, and thus resistance to PHS.

Key words: ABA responsiveness, allele, expression, pre-harvest sprouting, RT-PCR, *Triticum aestivum* L, *Vp-1*.

Introduction

Pre-harvest sprouting (PHS) of grain while it is still in the ear, usually in response to damp conditions, is due to an early disruption of seed dormancy (Bewley, 1997; Lenton, 2001; Appels *et al*., 2003; Finch-Savage and Leubner-Metzger, 2006). The main effects of PHS on wheat are a lower yield due to harvest losses and, more importantly, a reduction in end-product quality. Flour obtained from sprouted grains loses its viscosity due to starch breakdown, and baked goods from such flour are smaller in volume and have a compact, sticky crumb structure

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(Humphreys and Noll, 2002). Producers suffer great losses when wheat damaged by sprouting is sold at a discount; millers are faced with reduced flour yields and bakers encounter problems in processing and quality due to starch damage (Derera *et al*., 1977; Lenton, 2001).

A number of genes and QTLs involved in PHS tolerance or seed dormancy have been found and mapped in wheat (Flintham *et al*., 2002; Appels *et al*., 2003; Lohwasser *et al*., 2005). Among them, *Viviparous-1* (*Vp-1*) homologues play an important role in seed maturation, dormancy, and desiccation (McCarty *et al*., 1989, 1991; Giraudat *et al*., 1992; Bailey *et al*., 1999). The *Vp-1* gene is a major regulator of late embryo development in maize and inactivation of this locus leads to disruption of embryo maturation, resulting in the promotion of germination of embryos while still attached to the cob (vivipary) (McCarty *et al*., 1991). The phenotypes of *vp-1* mutants show that this locus performs two distinct functions: to promote embryo maturation and embryo dormancy and, simultaneously, to repress germination (McCarty *et al*., 1991; Hoecker *et al*., 1995). The *Arabidopsis ABI3* gene encodes a transcriptional factor homologous to maize *Vp-1* (McCarty *et al*., 1991; Giraudat *et al*., 1992) and acts with *ABI5* to control embryonic gene expression and seed sensitivity to ABA (Lopez-Molina *et al*., 2002; Nakashima *et al*., 2006). Mutants in the *Arabidopsis ABI3* gene result in a similar phenotype to that observed in maize *vp-1* embryos; the apical meristem is activated prematurely during embryo maturation, storage products do not accumulate, and the promoter of the germination-related *CAB3* gene is activated before seed shedding (Nambara *et al*., 1994). The *Arabidopsis ABI3* and maize *Vp-1* mutants do not have altered ABA levels in the embryo, but show reduced sensitivity to ABA, leading to precocious germination (Zhang *et al*., 2006*b*).

Orthologues of *Vp-1* have been cloned from rice (*Osvp1*; Hattori *et al*., 1994), wild oat (*AfVp1*; Jones *et al*., 1997), and several dicotyledonous species such as *Arabidopsis* (*ABI3*; Giraudat *et al*., 1992) and poplar (*PtABI3*; Rohde *et al*., 2002). Biochemical analyses have shown that the Vp1 protein functions as a transcription factor, containing both transcriptional activation and DNA binding domains (McCarty *et al*., 1991; Suzuki *et al*., 1997). Comparisons with orthologues from other species identified four highly conserved amino acid domains: A1, which is an acidic region at the N-terminus of the protein, and three basic domains designated B1, B2, and B3 (Giraudat *et al*., 1992). The B1 and B2 domains play important roles in nuclear location and non-specific interactions with other proteins and enhance the DNA binding activity of other transcription factors (Giraudat *et al*., 1992; Ezcurra *et al*., 2000; Nakamura and Toyama, 2001), whereas the C-terminal B3 domain shows highly cooperative sequence-specific DNA binding to the RY/Sph *cis* elements present in the promoter regions of related genes (Suzuki *et al*., 1997). The multiple domains of ABI3 enable it to function either as an activator or a repressor depending on the promoter context (Zhang *et al*., 2006*b*). The N-terminal A1 domain is responsible for ABA-dependent co-activation and repression activities (Hoecker *et al*., 1995; Carson *et al*., 1997), whereas the C-terminal B3 domain is essential for activation of a subset of genes (Carson *et al*., 1997).

Vp-1 homologues were mapped in wheat to the long arms of group 3 chromosomes at a location which is conserved relative to the *Vp-1* gene in maize and the *Osvp1* gene in rice (Bailey *et al*., 1999). The *Vp-1* homologues from wheat show the same four highly conserved domains that are present in the *Avena fatua*, rice, and maize proteins (Nakamura and Toyama, 2001). The structure and expression of the three *Vp-1* homologues in common wheat have been determined, showing that each has the potential to encode a full-length functional protein. However, alternative splicing of pre-mRNA leads to a diverse population of RNAs, which in most cases encode aberrant translation products (McKibbin *et al*., 2002). Comparison of the transcript structures of wheat and closely related wild and ancestral species suggest that mis-splicing of *Vp-1* genes might occur before the evolution and domestication of common wheat (McKibbin *et al*., 2002). Further studies of *Vp-1* transcript structure within the Triticeae tribe revealed that alternative splicing is also present in barley, rye, *Brachypodium pinnatum*, *Agropyron repens*, *Elymus repens*, and *Thinopyrum scripeum*, with *Triticum* and *Aegilops* having similar alternative spliced forms with highly conserved exon/intron borders for intron 1 (Wilkinson *et al*., 2005). However, only one transcript exists for *Vp-1*, and its level in mature embryos of dormant and non-dormant wheat cultivars showed positive correlations with seed dormancy and embryo sensitivity to ABA (Nakamura and Toyama, 2001). In *Avena fatua*, expression of *AfVp1* is controlled by the interaction between the environment and genotype, with a close correlation observed between *AfVp1* mRNA levels and seed dormancy (Jones *et al*., 1997). Transgenic wheat seeds expressing the *AfVp1* cDNA also showed increased dormancy and tolerance to PHS (McKibbin *et al*., 2002).

In order to exploit novel genetic resources of *Vp-1* for PHS tolerance in bread wheat, the *Vp-1* homologues were isolated from three common wheat cultivars which respond differently to ABA and tolerate PHS differently using genome-specific primers. Sequence analyses indicated that two new *Vp-1* alleles associated with PHS tolerance were explored on the 3BL chromosome. The expression patterns of all three *Vp-1B* alleles at different stages of seed development were also compared and the responses of the three cultivars to ABA treatment were determined.

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Materials and methods

Plant materials

Three common wheat cultivars, Yongchuanbaimai (a typical PHS-tolerant Chinese landrace, with a germination index of 0.14 averaged from three locations, i.e. Beijing, Anyang, and Zhenzhou in the 2005–2006 wheat season), Xinong 979 (a PHS-tolerant cultivar with a germination index of 0.50), and Zhongyou 9507 (a PHS-susceptible cultivar with a germination index of 0.84), were used in this study. In Beijing, they were planted at the CAAS experimental station, each in three rows, in late September 2005. The trials were kept free of weeds and diseases by two applications of broad-range herbicides and fungicides. Ears were harvested at 25 DAP (days after pollination), 30 DAP, and 35 DAP, respectively, and then frozen in liquid nitrogen and stored at −70 °C for further analysis.

ABA treatment

Embryos from the three cultivars were isolated at the dough stage (35 DAP) under sterilized conditions and stratified on filter paper saturated with water or 30 μM ABA solution in Petri dishes at 15 °C in darkness for 2, 4, and 7 d. RNA was extracted from untreated and treated embryos at different time points of treatment.

Primer design

Gene-specific primers were designed based on the DNA sequence alignment of the three homologues *Vp-1A*, *Vp-1B*, and *Vp-1D* available in GenBank (http://www.ncbi.nlm.nig.gov) under the accession numbers AJ400712, AJ400713, and AJ400714, respectively. Twelve pairs of primers, Vp-$1AF_1/R_1$, Vp-$1AF_2/R_2$, Vp-$1AF_3/R_3$, Vp-$1AF_4/R_4$, Vp-$1BF_1/R_1$, Vp-$1BF_2/R_2$, Vp-$1BF_3/R_3$, Vp-$1BF_4/R_4$, Vp-$1DF_1/R_1$, Vp-$1DF_2/R_2$,Vp-$1DF_3/R_3$, and Vp-$1DF_4/R_4$, were designed to amplify fragments of the *Vp-1A*, *Vp-1B*, and *Vp-1D* genes (Table 1). Another three pairs of genome-specific primers, RTVp-1AF/R, RTVp-1BF/R, and RTVp-1DF/R (Table 1), were designed to perform RT-PCR of the *Vp-1A*, *Vp-1B*, and *Vp-1D* genes, respectively. The wheat *ACTIN* gene was included as an internal control in each reaction in order to normalize the expression level of *Vp-1* genes using a primer pair designed to amplify a 410 bp product (Table 1).

DNA extraction and PCR amplification

Genomic DNA was isolated from kernels using the method described by Gale *et al.* (2001). For each genotype, three samples (two from individual seeds and one from a composite sample of three to six seeds) were amplified in order to verify the purity of the sample. PCR reactions were performed in an MJ Research PTC-200 thermal cycler in a total volume of 50 μl including10× PCR buffer, 125 μM each of dNTP, 8 pmol of each primer, 2.0 units of rTaq polymerase, and 100 ng of template DNA. The following conditions for PCR amplification were 94 °C for 5 min, followed by 36 cycles of 94 °C for 1 min, 53–66 °C for 1 min, and 72 °C for 1 min, with a final extension of 72 °C for 10 min. Amplified PCR fragments were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized using UV light.

RNA isolation and semi-quantitative RT-PCR analysis

Total RNA was extracted from embryos at different developmental stages as described by Chang *et al.* (1993). RNA concentration and quality were determined by spectrophotometer at 260 nm and by the A260:A280 ratio, respectively. RNA integrity was assessed by comparing the relative intensities of the 28S and 18S rRNA bands in 1.2% (w/v) agarose gels containing 2.2 M formaldehyde. cDNA was synthesized from 5 μg of the total RNA using M-MLV reverse transcriptase (TaKaRa) with random hexamer primer oligo $d(T)_{18}$ according to the manufacturer's instructions.

Semi-quantitative RT-PCR reactions were performed in an MJ Research PTC-200 thermal cycler in a total volume of 25 μl, using the protocol in the instruction manual of the GC PCR kit (Clontech), including 1 μl of the cDNA template. The reaction conditions were 94 °C for 5 min, followed by 36 cycles of 94 °C for 1 min, 60–68 °C for 1 min, and 72 °C for 1 min, with a final extension of 72 °C for 10 min. The RT-PCR products were separated on a 2.0% agarose gel. Values were normalized with the amplification rate of the *ACTIN* gene as a constitutively expressed internal control. Three replicates were performed for each sample.

DNA sequencing and analysis

The PCR and cloned products were sequenced from both strands by Shanghai Sangon Biological Technology Co. Ltd (http://www.sangon.com). Sequence analysis and characterization were performed using software DNAMAN (http://www.lynon.com).

Table 1. *The primer sets used for amplification of the genomic sequence and semi-quantitative RT-PCR analysis of* Vp-1A*,* Vp-1B*, and* Vp-1D *genes in common wheat*

Primer set	Upstream (5′−3′)	Downstream (5′−3′)	Annealing (°C)	Fragment size (bp)
Vp-$1AF_1/R_1$	ATCCAAACCGGCGGCTTCCCTCAAGA	CAAAATCGATCGATGGGAGTACTAG	56	1108
Vp-$1AF_2/R_2$	AGGACATCGGCACATCTCA	CTGGTCAGTTTGCAACATGCAAC	53	912
Vp-$1AF_3/R_3$	TGGAGATCCGGCAGGGAGAG	CCAGAGGCCTCCCCAGCCA	67	1253
Vp-$1AF_4/R_4$	GAATGAGCTGCAGGAGGGTGA	GCAATGCATGACTAACTAGG	58	1207
Vp-$1BF_1/R_1$	ATCCAAACCGGCGGCTTCCCTCAAGA	CTTACCGGTACCGCATGCTCCAG	60	1031
Vp-$1BF_2/R_2$	AGGACATCGGCACATCTCA	CAAAATGGCAGCAACTGATCAGTTC	55	960, 1153, 877
Vp-$1BF_3/R_3$	ATGGACGCCTCCGCCGGCTC	CTGCTGCTGCAGGCACGACAA	65	1227
Vp-$1BF_4/R_4$	CAATGAGCTGCAGGAGGGTGA	ATCATCCCTAACTAGGGCTACG	66	911
Vp-$1DF_1/R_1$	ATCCAAACCGGCGGCTTCCCTCAAGA	GAACGTGCGTGTCCCACACAC	60	1214
Vp-$1DF_2/R_2$	AGGACATCGGCACATCTCA	CCGCCTTATATTTTGATACGC	60	1025
Vp-$1DF_3/R_3$	TGGAGATCCGGCAGGGAGAG	CTG GCC CTG GAC GGC ATGC	67	1282
Vp-$1DF_4/R_4$	GAATGGCTGCAGGAGGGTGA	CCGATAGCTACTTTAGTATCAC	58	1033
RTVp-1AF/R	ATCCAAACCGGCGGCTTCCCTCAAGA	GCTTGGCTAGATCCTGTTGCGCT CTC	68	672
RTVp-1BF/R	ATCCAAACCGGCGGCTTCCCTCAAGA	CTTGTGCTTGGCTAGATC CTGTTGA	60	672
RTVp-1DF/R	ATCCAAACCGGCGGCTTCCCTCAAGA	CTTCTCTTTGCAACCACCGTCTTG	62	672
ACTIN up/down	GTTTCCTGGAATTGCTGATCGCAT	CATTATTTCATACAGCAGGCAAGC	62	410

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Results

Isolation and sequence analysis of the three Vp-1 *homologues in cultivars differing in PHS tolerance*

Full sequences of the three *Vp-1* homologues were isolated using genome-specific primers (Table 1). Sequence alignment showed that only the *Vp-1B* gene gave polymorphic fragments in the three cultivars with the primer set Vp-1BF_2/R_2, whereas no polymorphism was detected in the *Vp-1A* and *Vp-1D* genes (data not shown). The *Vp-1B* gene in Yongchuanbaimai (which showed consistently higher PHS resistance over years and locations) was 4227 bp with a 193 bp insertion in the third intron, whereas that in the newly released PHS-resistant cultivar Xinong 979 was 3942 bp with an 83 bp deletion in the same intron. Neither of these mutations was present in Zhongyou 9507 which is susceptible to PHS (Fig. 1). Comparison with the TIGR plant repeat database (http://

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Y  CTCTGCATCACTGTGGGACACGCATGCTCCTTTCCCAATTGGGCAAGTGGGTCATATGAA  180
Z  CTCTGCATCACTGTGGGACACGCATGCTCCTTTCCCAATTGGGCAAGTGGGTCATATGAA  178
X  CTCTGCATCACTGTGGGACACGCATGCTCCTTTCCCAATTGGGCAAGTGGGTCATATGAA   38

Y  TACATATGATGGGAATCCGGGAGCAAAATTGCTTTACACACGACGGTTAGTGCACGACGC  240
Z  TACATATGATGGGA.............................................  192
X   TACATATGATGGGA.............................................   52

Y  TTGCACGATTCTCCCTCCCCAACAGGGCTCCTCCACACACGGTGGCTGCTGGTGCTTTTT  300
Z  .............................................................  192
X  .............................................................   52

Y  TTAATTGAGAATCGGTCGCTGCTGGTGCTGCCACGTATGAATCGTTCATGCATCGGCCGT  360
Z  .............................................................  192
X  .............................................................   52

Y  GCAGGCATCGTCTATCTAGCAACGCTCATCCGGGAGCCAAATAGTAGCATGCATTCGTAG  420
Z  ..........................ATCCGGGAGCCAAATAGTAGCATGCATTCGTAG  225
X  ..........................ATCCGGGAGCCAAATAGTAGCATGCATTCGTAG   85

Y  TGCGCTCTCTCTATATATACGTGAACGGGCGGGCATGCAGAAATTTTGTTTTCGCATCGT  480
Z  TGCGCTCTCTCTATATATACGTGAACGGGCGGGCATGCAGAAATTTTGTTTTCGCATCGT  285
X  TGCGCTCTCTCTATATATACGTGAACGGGCGGGCATGCAGAAATTTTGTTTTCGCATCGT  145

Y  CTCTTGGTTCACTCCTTGTTAGCATCACTCTAGACAGTATATTATCATAATGAAGTGGTG  540
Z  CTCTTGGTTCACTCCTTGTTAGCATCACTCTAGACAGTATATTATCATAATGAAGTGGTG  345
X  CTCTTGGTTCACTCCTTGTTAGCATCACTCTAGACAGTATATTATCATAATGAAGTGGTG  205

Y  TTTCTGATACCATACTTCCTATTTTTCCGTCTTCTTCTTCTTCTTCCGAAGTGTATCATA  600
Z  TTTCTGATACCATACTTCCTATTTTTCCGTCTTCTTCTTCTTCTTCCGAAGTGTATCATA  405
X  TTTCTGATACCATACTTCCTATTTTTCCGTCTTCTTCTTCTTCTTCCGAAGTGTATCATA  265

Y  TGTTGCTAGTTACTCCCTCCGTAAACTAATATAAGAGCGTTTAGATTACTATTTTAGTGA  660
Z  TGTTGCTAGTTACTCCCTCCGTAAACTAATATAAGAGCGTTTAGATTACTATTTTAGTGA  465
X  TGTTGCTAGT..................................................  275

Y  TCTAAATGCTCTTATATTAGTTTACAGAGGGAGTAATTAGCTAGAGCTATCAAAATGAGA  720
Z  TCTAAATGCTCTTATATTAGTTTACAGAGGGAGTAATTAGCTAGAGCTATCAAAATGAGA  525
X  ..............................TAATTAGCTAGAGCTATCAAAATGAGA  302

Y  AAAAAAGAACTAGCTAGTTTGTATTCTGCATGCATGATAACAAATTGCTGGTACAATGAT  780
Z  AAAAAAGAACTAGCTAGTTTGTATTCTGCATGCATGATAACAAATTGCTGGTACAATGAT  585
X  AAAAAAGAACTAGCTAGTTTGTATTCTGCATGCATGATAACAAATTGCTGGTACAATGAT  362

Y  GGCTCTTGATTTTTCATGCATGCAGATTTTGGCCCAACAACAAGAGCAGAATGTATCTTC  840
Z  GGCTCTTGATTTTTCATGCATGCAGATTTTGGCCCAACAACAAGAGCAGAATGTATCTTC  645
X  GGCTCTTGATTTTTCATGCATGCAGATTTTGGCCCAACAACAAGAGCAGAATGTATCTTC  422

Y  TAGAGAACACTGGTGAGAGAAGAGAAGCAAACTTGCCTGATACAATTTTGAAACCAAATA  900
Z  TAGAGAACACTGGTGAGAGAAGAGAAGCAAACTTGCCTGATACAATTTTGAAACCAAATA  705
X  TAGAGAACACTGGTGAGAGAAGAGAAGCAAACTTGCCTGATACAATTTTGAAACCAAATA  482

Y  TGTTGCTAGCTTCCCTTGTGTTCACAAAACGTTTCTTCTGCTTGACCTTGTAGGTGACTT  960
Z  TGTTGCTAGCTTCCCTTGTGTTCACAAAACGTTTCTTCTGCTTGACCTTGTAGGTGACTT  765
X  TGTTGCTAGCTTCCCTTGTGTTCACAAAACGTTTCTTCTGCTTGACCTTGTAGGTGACTT  542

Y  TGTTCGGTCCAATGAGCTGCAGGAGGGTGATTTCATCGTGCTTTACTCTGATGTCAAGTC 1020
Z  TGTTCGGTCCAATGAGCTGCAGGAGGGTGATTTCATCGTGCTTTACTCTGATGTCAAGTC  825
X  TGTTCGGTCCAATGAGCTGCAGGAGGGTGATTTCATCGTGCTTTACTCTGATGTCAAGTC  572
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Fig. 1. Alignment of partial wheat *Vp-1B* genomic sequence in PHS-resistant cultivars Yongchuanbaimai (Y) and Xinong 979 (X), and PHS-susceptible cultivar Zhongyou 9507 (Z). Sequence alignment indicated that, compared with Z, Y has a 193 bp insertion, while X has an 83 bp deletion. Sequences underlined indicated the postulated exon–intron boundaries.

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tigrblast.tigr.org) revealed that the 193 bp insertion was homologous with the maize and barley gypsy/Ty3 retrotransposon Tekay (AF050455 and AY040832) with a probability of 0.92, whereas the 83 bp deleted region had high similarity to a transposon-like sequence in rice (AY090462.1), with a probability of 0.9999. These two new *Vp-1B* alleles from the PHS-tolerant lines were designated *Vp-1Bb* and *Vp-1Bc*, respectively, according to the 2005 Supplement of the Wheat Gene Catalogue (McIntosh *et al*., 2005). Sequence analysis showed that the *Vp-1Bb* and *Vp-1Bc* alleles had 95.43% and 97.89% similarity to the sequence of AJ400713 (*Vp-1Ba*) at the nucleotide level. These two sequences were deposited in the GenBank under accession numbers of DQ517493 and DQ517494.

Expression characterization of Vp-1A*,* Vp-1B*, and* Vp-1D *in three cultivars differing in PHS tolerance*

In order to define the expression patterns of the three *Vp-1* homologues and their relationship with PHS tolerance, semi-quantitative RT-PCR analysis was carried out to determine the expression levels of *Vp-1A*, *Vp-1B*, and *Vp-1D* in the three wheat cultivars differing in PHS tolerance, using the *ACTIN* gene as an internal control. A set of transcripts for *Vp-1A, Vp-1B*, and *Vp-1D* was detected in 35 DAP embryos but only one of these transcripts had the capacity to encode the correct protein product (data not shown). Furthermore, no differences were observed in the transcript abundances of *Vp-1A* and *Vp-1D* between the PHS-susceptible and -resistant cultivars (Fig. 2). Although all the three *Vp-1B* alleles tested in this study had alternatively spliced transcripts, different expression levels of the correctly spliced transcripts for *Vp-1Ba*, *Vp-1Bb*, and *Vp-1Bc* were observed, with *Vp-1Bb* being the most abundant and *Vp-1Ba* the least abundant (Fig. 3).

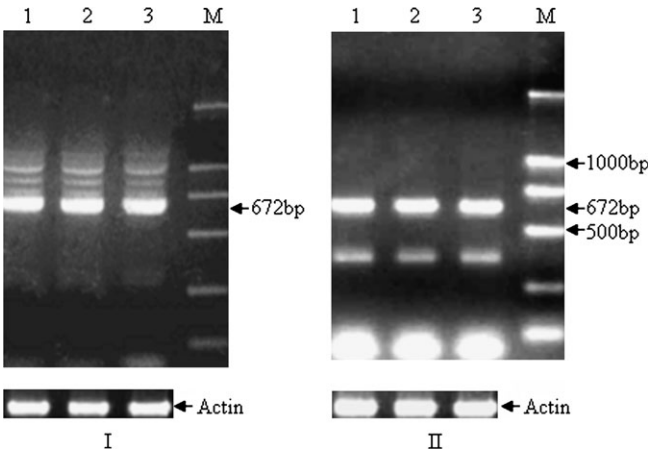

Fig. 2. Semi-quantitative RT-PCR analysis of *Vp-1A* and *Vp-1D* in 35 DAP embryos of three cultivars differing in PHS tolerance. Lanes: M, D-2000 DNA marker; 1, Zhongyou 9507; 2, Xinong 979; 3, Yongchuanbaimai. (I) Comparison of the transcript levels of *Vp-1A* in three cultivars; (II) comparison of the transcript levels of *Vp-1D* in three cultivars.

Semi-quantitative RT-PCR was also employed to determine the expression patterns of the *Vp-1B* alleles at different seed developmental stages (25, 30, and 35 DAP). As shown in Fig. 3, no transcripts of the three *Vp-1B* alleles were detected in 25 DAP embryos and only single and presumably correctly spliced transcripts of each were present in 30 DAP embryos, with *Vp-1Bc* having the highest transcript abundance and *Vp-1Ba* the lowest. Although mis-spliced transcripts were present in 35 DAP embryos, the majority of the transcripts were correctly spliced. The *Vp-1Bb* and *Vp-1Bc* alleles had higher abundances of correctly spliced transcripts than *Vp-1Ba*, with the level being highest in the *Vp-1Bb* allele and lowest in *Vp-1Ba*, as observed previously by Nakamura and Toyama (2001). It was also observed that mis-spliced transcripts were present only at the late stage of seed development with the correctly spliced transcripts accumulating during the 25–35 DAP period which corresponds to the end of the exponential accumulation of kernel components. This was consistent with the development of seed dormancy and indicated that expression of the *Vp-1B* gene and its alleles might be developmentally regulated.

Expression characterization of three Vp-1B *alleles upon ABA treatment*

Semi-quantitative RT-PCR was used to determine the expression of the three *Vp-1B* alleles in 35 DAP embryos stratified with or without 30 μM ABA solution in order to investigate the effects of insertion or deletion in the third intron on ABA responsiveness. As shown in Fig. 4, soaking in water for 2 d in the absence of ABA was sufficient to reduce the expression of all the three *Vp-1B* alleles to below detectable levels, indicating that the embryos developed into autotrophic seedlings. By contrast, in the presence of 30 μM ABA, all three alleles showed detectable transcripts in 2-d-old germinating embryos with the expression level of *Vp-1Bb* higher than that of the other two. However, the transcript levels of both *Vp-1Bb* and *Vp-1Bc* then decreased, falling to below detectable levels after 4 d of treatment with 30 μM ABA, whereas the level of *Vp-1Ba* transcripts remained unchanged, suggesting that the three alleles differed in their response to ABA exposure.

Discussion

The effect of insertion and deletion on expression of Vp-1B *and PHS tolerance*

Compared with *Vp-1Ba*, the new allele *Vp-1Bb* had a 193 bp insertion in the third intron at position 2496 bp of AJ400713 (*Vp-1Ba*), whereas *Vp-1Bc* had an 83 bp

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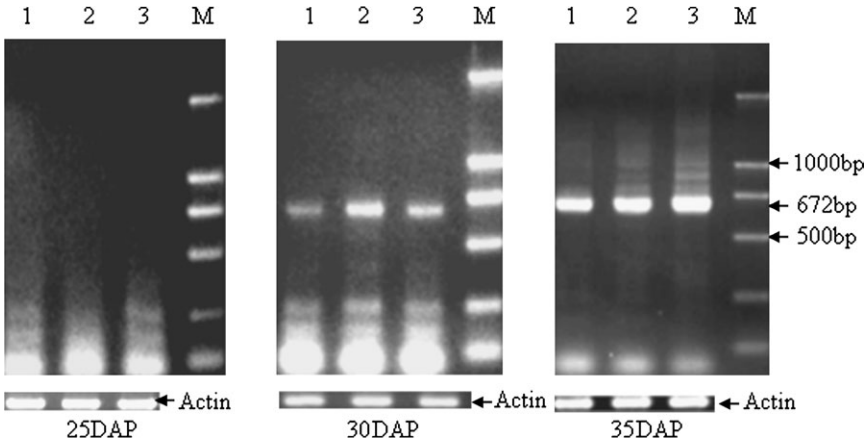

Fig. 3. Semi-quantitative RT-PCR analysis of the *Vp-1B* alleles in 25, 30, and 35 DAP embryos. Lanes: M, D-2000 DNA marker; 1, Zhongyou 9507; 2, Xinong 979; 3, Yongchuanbaimai.

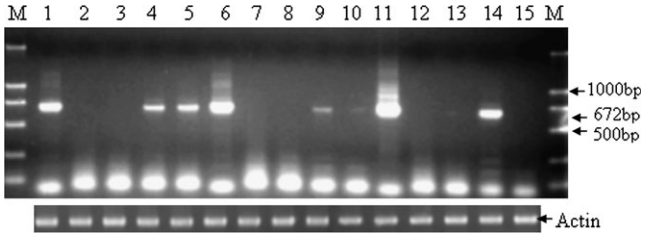

Fig. 4. Semi-quantitative RT-PCR analysis of *Vp-1B* alleles in 35 DAP embryos with or without ABA treatments. Lanes: M, D-2000 DNA marker; 1–5, Zhongyou 9507 (1, mature embryos; 2, mature embryos treated with water for 2 d; 3, mature embryos treated with water for 4 d; 4, mature embryos treated with 30 μM ABA solution for 2 d; 5, mature embryos treated with 30 μM ABA solution for 4 d); 6–10, Xinong 979 (6, mature embryos; 7, mature embryos treated with water for 2 d; 8, mature embryos treated with water for 4 d; 9, mature embryos treated with 30 μM ABA solution for 2 d; 10, mature embryos treated with 30 μM ABA solution for 4 d); 11–15, Yongchuanbaimai (11, mature embryos; 12, mature embryos treated with water for 2 d; 13, mature embryos treated with water for 4 d; 14, mature embryos treated with 30 μM ABA solution for 2 d; 15, mature embryos treated with 30 μM ABA solution for 4 d).

deletion at position 2712–2795 within the same intron. The inserted sequence had several putative exon–intron boundaries (GT–AG), while the deletion had characteristics typical of an intron, starting at 5′-GT and ending at 3′-AG (Fig. 1) (Yan *et al*., 2000; Alexei *et al*., 2003). The precise origin of the alternatively spliced forms is not known, but they may arise from inefficient splicing of potential intron–exon boundary sequences (Wilkinson *et al*., 2005). In addition, as discussed previously, the 193 bp insertion is highly homologous with the maize and barley gypsy/Ty3 retrotransposon Tekay (AF050455 and AY040832), whereas the 83 bp deletion is similar to a transposon-like sequence in rice (AY090462.1). In maize, many spontaneous waxy mutations are caused by transposable elements (Fedoroff *et al*., 1983; Wessler and Varagona, 1985; Wessler *et al*., 1987). It is therefore possible that the insertion and deletion may have occurred separately during the evolution of the alleles. Furthermore, a reverse repeat structure is present at a position from 2705 bp to 2801 bp in *Vp-1Ba* (Fig. 5), meaning that the insertion and deletion might affect the pre-mRNA structure and the expression level of the correctly spliced *Vp-1* transcripts. The insertion and deletion in the third intron region of *Vp-1B* was indeed associated with increased levels of correctly spliced *Vp-1B* transcript in 35 DAP mature embryos (Fig. 3). Moreover, further studies have shown that most of PHS-resistant genotypes have either the *Vp-1Bb* or the *Vp-1Bc* allele compared with PHS-susceptible genotypes, and that genotypes with the insertion are more tolerant of PHS than those with the deletion. The majority of genotypes with the 193 bp insertion were PHS-resistant landraces with germination rates below 8% (Y Yang, XL Zhao, LQ Xia, XM Chen, XC Xia, Z Yu, ZH He, unpublished results). In recent years, it has become clear that introns are functionally active participants of gene and genome function (Erkkilä and Ahokas, 2001; Fiume *et al*., 2004; Fu *et al*., 2005; Sjakste *et al*., 2006), they encode for regulatory elements with autocatalytic or alternative splicing activity, controlling gene transcription, and are sponsors of mobility by acting as either endonucleases or reverse transcriptases (Lewin, 2004). For example, in barley, a 126 bp insertion/deletion event (indel) in the 5′-region of intron III in the β-amylase gene is associated with allelic variants of the gene-encoding enzymes of correspondingly low or high thermo-stability (Erkkilä and Ahokas, 2001), and deletions in the promoter region and first intron of the *waxy* gene similarly result in decreased gene expression and reduced amylose levels (Domon *et al*., 2002; Patron *et al*., 2002).

McKibbin *et al*. (2002) reported that correctly spliced transcripts represented a small proportion of the wheat *Vp-1* mRNA population with only three of the seven

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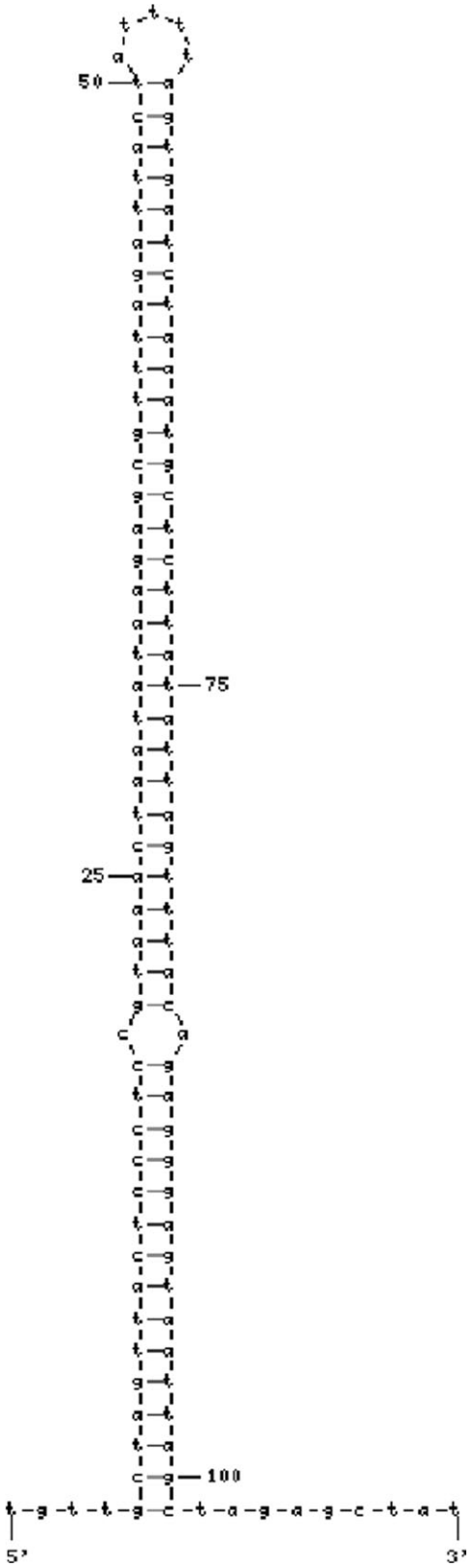

Fig. 5. Reverse repeat structures at position 2705–2801 bp of *Vp-1Ba*.

TaVp-1 cDNA clones studied in detail having the potential to encode full-length VP1 proteins. Furthermore, of the 84 random RT-PCR clones sequenced, 69% were shown to be properly spliced and from the B genome (Wilkinson *et al*., 2005). In this study, genome-specific primers were used to show that mis-spliced transcripts from the B genome are present only at the late seed developmental stage (35 DAP), with the majority of the transcripts being correctly spliced. Furthermore, the levels of correctly spliced transcripts increased during seed development, suggesting that alternative splicing might also be developmentally regulated. It was also shown that the genotype with higher PHS tolerance had higher transcript abundance than a PHS susceptible genotype, which is consistent with the close correlation between transcript abundance and the level of seed dormancy as reported by Nakamura and Toyama (2001). Moreover, no differences in expression between *Vp-1A* and *Vp-1D* were observed in 35 DAP embryos of PHS-tolerant and -susceptible genotypes although alternative splicing also existed for *Vp-1A* and *Vp-1D* transcripts.

ABA sensitivity of the three Vp-1B *alleles*

ABA plays an important role in the adaptation of vegetative tissue to abiotic environmental stresses such as drought and high salinity as well as in seed maturation and dormancy (Gubler *et al*., 2005; Nakashima *et al*., 2006). Maize VP1 and *Arabidopsis* ABI3 are orthologous transcription factors that regulate key aspects of plant seed development and ABA signalling (Suzuki *et al*., 2003). Null alleles of *ABI3* and *Vp-1* resulted in loss of ABA sensitivity, leading to non-dormancy or vivipary in *Arabidopsis* and maize (McCarty *et al*., 1989; Nambara *et al*., 1994). Moreover, there is evidence that ABA levels in mature wheat embryos are similar in both PHS-sensitive and -resistant cultivars and that sprouting behaviour is related more to the extent of ABA responsiveness of embryos than to ABA levels (Walker-Simmons and Sesing, 1990). A positive correlation between the degree of seed dormancy, ABA sensitivity, and the level of *Vp-1* transcripts in wheat mature embryos was also observed by Nakamura and Toyama (2001). In the present study, a genotype with the *Vp-1Bb* allele was most sensitive to ABA treatment with transcript levels in mature embryos after 2 d exposure to ABA being higher than the other two genotypes. The transcript levels in this genotype then fell and were undetectable after an additional 2 d of treatment. Although the genotype with *Vp-1Ba* was less sensitive to ABA, it still showed comparable transcript levels and remained unchanged after the additional 2 d treatment. The ABA responsiveness of the genotype with the *Vp-1Bc* allele fell between those with the *Vp-1Ba* and *Vp-1Bb* alleles, consistent with its resistance to PHS. It is not known why the levels of *Vp-1Bb* fell dramatically at 4 d of treatment with ABA, but it could indicate that other regulators or

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microRNAs could degrade the *Vp-1* mRNA when it reached certain levels. Notably, ABI3 protein in germinating *Arabidopsis* seeds is known to be labile and an ABI3-interacting protein (AIP2) negatively regulates ABA signalling by targeting this protein for post-translational degradation (Zhang *et al*., 2006*b*). It has also been observed that ABA induces the accumulation of microRNA159 (miR159) in an ABI3-dependent fashion and that miRNA159 mediates the cleavage of MYB101 and MYB33 transcripts *in vitro* and *in vivo*. These two MYB transcription factors function as positive regulators of ABA responses as null mutants of them show hyposensitivity to ABA, suggesting that ABA-induced accumulation of miR159 is a homeostatic mechanism which directs MYB101 and MYB33 transcript degradation to desensitize hormone signalling during seed stress responses (Zhang *et al*., 2006*b*). In addition, the expression level of the *Vp-1Ba* genotype treated with ABA differed little at 2 d from that at 4 d, showing that *Vp-1Bb* and *Vp-1Bc* differ from *Vp-B1a* in the way they responded to ABA. While null mutants of *ABI3* and *Vp-1* in *Arabidopsis* and maize result in complete loss of ABA sensitivity, *Vp-1Ba* in wheat seems to retain partial responsiveness to ABA, although it is less sensitive to ABA than *Vp-1Bb* and *Vp-1Bc*. Embryos of Yongchuanbaimai (*Vp-1Bb*) were more sensitive to ABA than those of Zhongyou 9507 (*Vp-1Ba*) (Zhang *et al*., 2006*a*). Considering that the 5′-UTR negatively regulates quantitative and spatial expression of *ABI3* (Ng *et al*., 2004), it would be interesting to investigate if any differences in the promoter region of these *Vp-1B* alleles exist which could affect their expression level. Nevertheless, the present study clearly demonstrates that the insertion and deletion in the *Vp-1B* gene could enhance its expression and ABA sensitivity, and thus resistance to PHS.

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References

Alexei F, Scott R, Larisa F, Walter G. 2003. Mystery of intron gain. *Genome Research* **13,** 2236–2241.

Appels R, Francki M, Chibbar R. 2003. Advances in cereal functional genomics. *Functional and Integrative Genomics* **3,** 1–24.

Bailey PC, McKibbin RS, Lenton JR. 1999. Genetic map location for orthologous VP1 genes in wheat and rice. *Theoretical and Applied Genetics* **98,** 281–284.

Bewley JD. 1997. Seed germination and dormancy. *The Plant Cell* **9,** 1055–1066.

Carson CB, Hattori T, Rosenkrans L, Vasil V, Peterson PA, McCarty DR. 1997. The quiescent/colorless alleles of viviparous1 show that the conserved B3 domain of Vp1 is not essential for ABA-regulated gene expression in the seed. *The Plant Journal* **12,** 1231–1240.

Chang S, Puryear J, Cairney J. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter* **11,** 113–116.

Derera NF, Bhatt GM, McMaster GJ. 1977. On the problem of pre-harvest sprouting of wheat. *Euphytica* **26,** 299–308.

Domon E, Fujita M, Ishikawa N. 2002. The insertion/deletion polymorphisms in the *waxy* gene of barley genetic resources from East Asia. *Theoretical and Applied Genetics* **104,** 132–138.

Erkkilä MJ, Ahokas H. 2001. Special barley β-amylase allele in a Finnish landrace line HA52 with high grain enzyme activity. *Hereditas* **134,** 91–95.

Ezcurra I, Wycliffe P, Nehlin L, Ellerstrom M, Rask L. 2000. Transactivation of the *Brassica napus* napin promoter by ABI3 requires interaction of the conserved B2 and B3 domains of ABI3 with different cis-elements: B2 mediates activation through an ABRE, whereas B3 interacts with an RY/G-box. *The Plant Journal* **24,** 57–66.

Fedoroff N, Wessler S, Shure M. 1983. Isolation of the transposable maize controlling elements Ac and Ds. *Cell* **85,** 235–242.

Finch-Savage WE, Leubner-Metzger G. 2006. Seed dormancy and the control of germination. *New Phytologist* **171,** 501–523.

Fiume E, Christou P, Giani S, Beviarion D. 2004. Introns are key regulatory elements of rice tubulin expression. *Planta* **218,** 693–703.

Flintham JE, Adlam R, Bassoi M, Holdsworth M, Gale M. 2002. Mapping genes for resistance to sprouting damage in wheat. *Euphytica* **126,** 39–45.

Fu D, Szücs P, Yan L, Helguera M, Skinner JS, von Zitzewitz J, Hayes PM, Dubkovskii J. 2005. Large deletions within the first intron in VRN-1 are associated with spring growth habit in barley and wheat. *Molecular Genetics and Genomics* **273,** 54–65.

Gale KR, Ma W, Zhang W, Rampling L, Hill AS, Appels R, Morris P, Morrel M. 2001. Simple high-throughput DNA markers for genotyping in wheat. In: Eastwood R, Hollamby G, Rathgen A, Gororo N, eds. *Proceesings of 10th Assembly of the Wheat Breeding Society of Australia* 26–31.

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM. 1992. Isolation of the Arabidopsis ABI3 gene by positional cloning. *The Plant Cell* **4,** 1251–1261.

Gubler F, Millar AA, Jacobsen JV. 2005. Dormancy release, ABA and pre-harvest sprouting. *Current Opinion in Plant Biology* **8,** 183–187.

Hattori T, Terada T, Hamasuna ST. 1994. Sequence and functional analysis of the rice gene homologous to maize *Vp1*. *Plant Molecular Biology* **24,** 805–810.

Hoecker U, Vasil IK, McCarty DR. 1995. Integrated control of seed maturation and germination programs by activator and repressor functions of Viviparous-1 of maize. *Genes and Development* **9,** 2459–2469.

Humphreys DG, Noll J. 2002. Methods for characterization of preharvest sprouting tolerance in a wheat breeding program. *Euphytica* **126,** 61–65.

Jones HD, Peters NC, Holdsworth MJ. 1997. Genotype and environment interact to control dormancy and differential expression of the VIVIPAROUS 1 homologue in embryos of *Avena fatua*. *The Plant Journal* **12,** 911–920.

Lenton J. 2001. Opportunities for the manipulation of development of temperate cereals. *Advances in Botanical Research* **34,** 127–164.

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Lewin B. 2004. *Genes VIII*. Upper Saddle River, NJ: Pearson, Prentice Hall.

Lohwasser U, Röder MS, Börner A. 2005. QTL mapping of the domestication traits pre-harvest sprouting and dormancy in wheat (*Triticum aestivum* L.). *Euphytica* **143,** 247–249.

Lopez-Molina L, Mongrand S, McLachlin DT, Chait BT, Chua NH. 2002. ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. *The Plant Journal* **32,** 317–328.

McCarty DR, Carson CB, Stinard PS, Robertson DS. 1989. Molecular analysis of *VIVIPAROUS-1*: an abscisic acid insensitive mutant of maize. *The Plant Cell* **1,** 523–532.

McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK. 1991. The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. *Cell* **66,** 895–905.

McIntosh RA, Devos KM, Dubcovsky J, Rogers WJ, Morris CF, Appels R, Anderson OA. 2005. *Catalogue of gene symbols for wheat: 2005 supplement – DNA markers.* Published online at: http://wheat.pw.usda.gov/ggpages/awn/51/Textfiles/WGC.html.

McKibbin RS, Wilkinson MD, Bailey PC, Flintham JE, Andrew LM, Lazzeri PA, Gale MD, Lenton JR, Holdworth MJ. 2002. Transcripts of *Vp-1* homologues are misspliced in modern wheat and ancestral species. *Proceedings of the National Academy of Sciences, USA* **99,** 10203–10208.

Nakamura S, Toyama T. 2001. Isolation of a *VP1* homologue from wheat and analysis of its expression in embryos of dormant and non-dormant cultivars. *Journal of Experimental Botany* **52,** 875–876.

Nakashima K, Fujita Y, Katsura K, Maruyama K, Narusaka Y, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. 2006. Transcriptional regulation of ABI3- and ABA-responsive genes, including RD29B and RD29A in seeds, germinating embryos and seedlings of Arabidopsis. *Plant Molecular Biology* **60,** 50–68.

Nambara E, Keith K, McCourt P, Naito S. 1994. Isolation of an internal deletion mutant of the *Arabidopsis thaliana* ABI3 gene. *Plant Cell Physiology* **35,** 509–513.

Ng DW-K, Chandrasekharan MB, Hall TC. 2004. The 5′ UTR negatively regulates quantitative and spatial expression from *ABI* promoter. *Plant Molecular Biology* **54,** 25–38.

Patron NJ, Smith AM, Fahy BF, Hylton CM, Naldrett MJ, Rossnagel BG, Denyer K. 2002. The altered pattern of amylose accumulation in the endosperm of low-amylose barley cultivars is attributable to a single mutant allele of granule-bound starch synthase I with a deletion in the 5′-non-coding region. *Plant Physiology* **130,** 190–198.

Rohde A, Prinsen E, De Rycke, Engler G, Van MM, Boerjan W. 2002. PtABI3 impinges on the growth and differentiation of embryonic leaves during bud set in poplar. *The Plant Cell* **14,** 1885–1901.

Sjakste TG, Zhuk AF. 2006. Novel haplotype description and structural background of the eventual functional significance of the barley β-amylase gene intron III rearrangements. *Theoretical and Applied Genetics* **113,** 1063–1079.

Suzuki M, Kao CY, McCarty DR. 1997. The conserved B3 domain of VIVIPAROUS1 has a cooperative DNA binding activity. *The Plant Cell* **9,** 799–807.

Suzuki M, Ketterling MG, Li QB, McMarty DR. 2003. Viviparous1 alters global gene expression patterns through regulation of abscisic acid signaling. *Plant Physiology* **132,** 1664–1677.

Walker-Simmons M, Sesing J. 1990. Temperature effects on embryonic abscisic acid levels during development of wheat grain dormancy. *Journal of Plant Growth Regulation* **9,** 51–56.

Wessler SR, Baran G, Varagona MJ. 1987. The maize transposable Ds element is spliced from RNA. *Science* **237,** 916–918.

Wessler SR, Varagona MJ. 1985. Molecular basis of mutations at the *waxy* locus of maize: correlation with the fine structure genetic map. *Proceedings of the National Academy of Sciences, USA* **82,** 4177–4182.

Wilkinson M, Lenton J, Holdsworth M. 2005. Transcripts of *VP-1* homologues are alternatively spliced within the Triticeae tribe. *Euphytica* **143,** 243–246.

Yan L, Bhave M, Fairclough R, Konik C, Rahman S, Appels R. 2000. The gene encoding granule-bound starch synthases at the waxy loci of the A, B, and D progenitors of common wheat. *Genome* **43,** 264–272.

Zhang HP, Chang C, Xiao SH. 2006*a*. Proteomic analysis on abscisic acid signal transduction in embryo dormancy of wheat (*Triticum aestivum* L.). *Acta Agronomica Sinica* **32,** 690–697.

Zhang XR, Garreton V, Chua NH. 2006*b*. The AIP2 E3 ligase acts as a novel negative regulator of ABA signaling by promoting ABI3 degradation. *Genes and Development* **19,** 1532–1543.

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